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PRINCIPAL INVESTIGATOR: Roberto P. Garofalo, M.D.

CONTRACTING ORGANIZATION: University of Texas Medical Branch at Galveston
Galveston, TX 77555-0372

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Roberto P. Garofalo, M.D.

E-Mail: rpgarofa@utmb.edu

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INTRODUCTION

This project is in response to the Department of Defense Congressionally Directed Medical Research Programs, Investigator-Initiated Research Award and is addressing the topic area "Childhood asthma". The project focuses on respiratory syncytial virus (RSV), the single most important pathogen causing acute respiratory-tract infections in children. RSV infections are a major precipitating factor of wheezing in asthmatic children and have been linked to both the development and the severity of asthma. Our group has established a multidisciplinary and highly integrated pre-clinical and translational research program that focuses on the role of oxidative injury in the pathogenesis of severe RSV infections. We have discovered that in the course of RSV infections reactive oxygen species (ROS) are rapidly generated along with viral-mediated inhibition of protective antioxidant enzyme (AOE) genes in the lung. Thus, we propose a new molecular pathway by which respiratory viruses induce lung inflammation, with implication for novel therapeutic strategies of lower respiratory infections and virus-triggered precipitation of asthma attacks.

I. BODY

Hypothesis

Respiratory syncytial virus (RSV) is the single most important virus causing acute respiratory-tract infections in children and is a major cause of severe respiratory morbidity and mortality in elderly (1). Overall, the World Health Organization estimates that RSV is responsible for 64 million clinical infections and 160 thousand deaths annually worldwide (2). In addition to acute morbidity, RSV infections have been linked to both the development and the severity of asthma. We have shown that ROS are involved in the signaling transduction pathways that control inducible expression of chemokine and other inflammatory genes in response to RSV infection, yet blocking ROS production does not significantly increase viral replication in the lung and even decreases viral replication in cells (3-6). Recently, in the course of proteomics studies aimed to profile global protein expression we made two important discoveries: 1) RSV potently inhibits the expression of antioxidant enzyme (AOE) genes, including Glutathione S-transferases (GST), Superoxide dismutases (SOD) and catalase; 2) following RSV infection, expression of nuclear NF-E2 related factor-2 (Nrf2), which positively regulates basal and inducible expression of AOE genes is downregulated both in cells and in the lung (7), while Nrf3 which negatively regulates AOE gene expression (8) is induced in epithelial cells. **Our general hypothesis is that ROS production along with the inhibition of cytoprotective AOE expression lead to severe manifestations of RSV infection.**

Specific Aims

Specific Aim 1 - To determine the mechanism(s) of inhibition of AOE expression in the lung during the course of RSV infection by investigating the role of Nrf-dependent gene transcription. Using a well characterized murine model of experimental infection we will establish by real-time PCR and WB the expression profile, kinetics and cellular source of AOE in the lung over a period of 21 days following RSV inoculation (**1a**). To test our novel hypothesis that RSV inhibits AOE gene transcription by inhibiting Nrf2 expression and/or activating Nrf3 we will perform WB and EMSA studies of total lung or cell-specific nuclear proteins (**1b**).

Specific Aim 2 - To establish whether pharmacologic intervention aimed to increase Nrf2 activation in the airways or to supplement the antioxidant response via synthetic antioxidant mimetics results in protection from viral-induced lung injury and clinical disease. We will test the specific hypothesis that increasing the lung/airway antioxidant capacity, either by activating Nrf-2-ARE-mediated expression of endogenous AOE genes (**2a**) or by providing exogenous synthetic antioxidants mimetics (**2b**) may be used as a pharmacologic strategy to treat RSV infections. Using the murine model we will determine by established clinical-like parameters and pathophysiologic endpoints of airway dysfunction the effect of such pharmacologic treatments on experimental RSV infection. Markers of oxidation and oxidative-associated injury will be used as correlates of protection following treatment with Nrf-2 modulating compounds or antioxidants mimetics.

Specific Aim 3 – To analyze whether distinct AOE expression profile at the airway mucosal site can discriminate between infants with different severity of illness and/or degree of oxidative-associated injury following naturally-acquired RSV infections. In **3a**, the profile and relative abundance of AOE proteins present in nasopharyngeal secretions (NPS) collected from infants with RSV infections of different clinical severity will be analyzed by Western blots. NPS will be also tested for a panel of oxidative stress markers, including the lipid peroxidation products 8-isoprostane, malonaldehyde (MDA) and 4-hydrynonenal (4-HNE). In **3b,** we will apply our novel biofluids fractionation platform to analyze the NPS proteome by high resolution two-dimensional gel electrophoresis (2DE), and MALDI-TOF/TOF mass spectroscopy. These studies will determine whether viral-mediated inhibition of AOE expression, which we discovered in epithelial cells and - in preliminary experiments - in mouse lung, is associated with the most severe clinical manifestations of RSV infection in children, thus contributing to oxidative injury in the airways.

Key Research Accomplishments and Reportable Outcomes

During the past year of funding we have made significant progresses in this project, as they relate to studies in all three aims of the grant. The majority of these studies have been included in a recent paper that has been submitted to the prestigious Journal of Physiology. The paper has been recently peer-reviewed and we are preparing a revised version that we strongly feel will be accepted for publication. We have included the

manuscript as an appendix to this report. Our main results are summarized herein and we kindly refer to the Figures and Figure numbers of the above-mentioned manuscript.

The use of recombinant SOD and SOD mimetics has been explored as therapeutics in a variety of disease models either *in vitro* or *in vivo*. A number of SOD mimetics based around organo-manganese complexes have been developed. They include metalloporphyrin-based compounds, such as AEOL10113 and 10150, cyclic polyamine-based molecules, such as M40403 and 40419, and the salen compounds, such as EUK-8, -134 and -189, the latter ones possessing also significant catalase and peroxidase activity [Reviewed in (3)]. Although EUKs have been used in a variety of disease models, there is no reported literature about their use in models of viral infections. Therefore, in this study we tested whether treatment with the antioxidant mimetics EUK-8 and -189, which possess significant catalase and peroxidase activity, in addition to SOD, could restore antioxidant enzyme capacity in RSV-infected airway epithelial cells and thereby exert a protective effect against RSV-induced oxidative stress. A549 cells were treated with increasing concentration of EUK and infected with RSV. Cells were harvested at different time p.i. to measure AOE activity in the presence or absence of EUK treatment. RSV infection induced a progressive increase in SOD activity with a concomitant decrease in catalase and peroxidase activity (manuscript, Fig.1). EUK-8 treatment further increased SOD activity but, more importantly, reversed the loss of catalase and peroxidase activity observed in response to RSV infection, with the highest dose of EUK-8 increasing the latter two AOE activities above values of uninfected cells (Fig.1). Similar results were obtained in cells treated with EUK-189 (data not shown). In agreement with the observed reduction in ROS production, EUK treatment of airway epithelial cells significantly reduced the elevated cellular levels of the lipid peroxidation markers MDA and 8-isoprostane generated in response to RSV infection. This effect was observed even at the lowest dose of EUK used (manuscript, Fig. 3), indicating that antioxidant mimetic treatment can effectively counteract viral-induced cellular oxidative stress. Moreover, cells supernatants were collected to measure levels of various cytokine and chemokines by Bio-Plex assay. As shown in Fig. 4A of the attached manuscript, EUK administration caused a dose-dependent decrease in several cytokines, such as IL-6 and G-CSF, and chemokines, such as IL-8, RANTES, MIP-1β and IP-10. Similar results were obtained in SAE cells, normal human airway epithelial cells derived from cadaveric donor, which we have previously shown to behave very similarly to A549 cells in terms of chemokine/cytokine gene expression, transcription factor and signaling pathway activation, after RSV infection (2; 6; 11; 14; 26; 28; 35)(manuscript, Fig. 4B).

We have also shown that the antioxidants N-acetyl cysteine or dimethyl sulfoxide significantly reduce RSV-dependent serine phosphorylation of the NF-κB subunit p65, resulting in the inhibition of RSV-induced expression of several NF-κB-dependent genes, without affecting nuclear translocation (18). To determine whether EUK treatment was able to modulate viral-induced NF-κB and IRF-3 activation, A549 cells were treated with 100 μM EUK-8, infected with RSV and harvested at 6 and 15 h p.i. to prepare either total cell lysates or nuclear extracts. IRF-3 nuclear levels or cellular levels of p65 serine phosphorylation were assessed by Western blot. As shown in Fig. 5, EUK-8 treatment significantly reduced activation of both transcription factors, in particular at the 15 h time

point of infection. Taken together, these results indicate that antioxidant mimetic treatment can effectively modulate the strong pro-inflammatory cellular response induced by this viral infection.

Effects of antioxidant mimetics on viral replication. To determine whether antioxidant mimetic treatment of A549 cells affected viral replication, we used two different approaches, the first employing a direct cell-based plaque immunostaining assay (20), while the second used viral antigen detection by Western blot. As shown in Fig. 6A, there was no significant difference in the number of plaques between untreated and EUK-treated cells at the two lower concentrations (10 and 100 μM), while the highest one, both in EUK-8 and -189-treated cells, led to a significant reduction in the number of plaques detected by immunostaining of infected cells. To further characterize the antiviral activity of EUKs, we assessed the expression of RSV proteins by Western blot. A549 cells were treated with different concentrations of EUKs and infected with RSV. At 24 h post-infection, cells extracts were prepared and RSV proteins were detected by Western blot using a polyclonal antibody, as described (20). In RSV-infected cells, viral proteins, including G, N, P, and M, were expressed at comparable levels in untreated and EUK-treated cells using concentrations of 10 and 100 μM, whereas significant lower (EUK-189) or almost no expression (EUK-8) of RSV proteins was detected in infected cells treated with 500 μM of both compounds (Fig. 6B), confirming the data obtained by direct plaque assay staining. This represents a major new finding that could significantly impact the future therapy of viral bronchiolitis and RSV infection in particular.

CONCLUSIONS

We do not anticipate major changes in our research plan and will focus mainly on studies with modulation of viral replication and disease by treatment with EUKs.

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ANTIOXIDANT MIMETICS MODULATE OXIDATIVE STRESS AND CELLULAR SIGNALING IN RESPIRATORY SYNCYTIAL VIRUS INFECTION

Yashoda M. Hosakote [1], Nicolas Mautemps[1], Tianshuang Liu[1], Roberto P. Garofalo[1,2,3] and Antonella Casola[1,2,3,#]

Departments of Pediatrics[1], Microbiology and Immunology[2], Sealy Center for Vaccine Development[3], University of Texas Medical Branch, Galveston Texas

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Correspondence should be addressed at: Antonella Casola, Department of Pediatrics, 301 University Blvd., Galveston, TX 77555-0366, Fax (409) 772-1761; Tel. (409) 747-0581

E-mail: ancasola@utmb.edu

ABSTRACT

Respiratory syncytial virus (RSV) is one of the most common causes of bronchiolitis and pneumonia among infants and young children worldwide. In previous investigations, we have shown that RSV infection induces rapid generation of reactive oxygen species (ROS), which modulate viral-induced cellular signaling, and down-regulation of antioxidant enzyme (AOE) expression, resulting in oxidative stress *in vitro* and *in vivo*, which plays a pathogenetic role in RSV-induced lung disease. In this study, we determined whether pharmacologic intervention with synthetic catalytic scavengers could reduce RSV-induced proinflammatory gene expression and oxidative cell damage in an *in vitro* model of infection. Treatment of airway epithelial cells (AECs) with the salen-manganese complexes EUK-8 or EUK-189, which possess superoxide dismutase, catalase and glutathione peroxidase activity, strongly reduced RSV-induced ROS formation, by increasing cellular AOE enzymatic activity, and levels of the lipid peroxidation products F_2-8-isoprostane and malondialdehyde (MDA), which are markers of oxidative stress. Treatment of AECs with AOE mimetics also significantly inhibited RSV-induced cytokines and chemokines secretion, and activation of the transcription factors Nuclear Factor (NF)-κB and Interferon Regulatory Factor (IRF)-3, which orchestrate proinflammatory gene expression. Both EUKs were able to reduce viral replication, when used at high doses. These results suggest that increasing antioxidant cellular capacities can significantly impact RSV-associated oxidative cell damage and cellular signaling and could represent a novel therapeutic approach in modulating virus-induced lung disease.

Keywords: RSV, airway epithelial cells, antioxidant enzyme mimetics, oxidative stress

INTRODUCTION

RSV is the one of the most important cause of viral upper and lower respiratory tract infections in infants and young children. RSV is so ubiquitous in nature that it will infect 100% of children before the age of 2 (?). The number of children hospitalized each year in the US with viral LRTI has recently been estimated at > 200,000, with 500 deaths per year in children under age 5 years (21). Although the mechanisms of RSV-induced airway disease and associated long- term consequences remain incompletely defined, the lung inflammatory response is thought to play a fundamental role. Oxidative stress has been shown to play an important role in the pathogenesis of both acute and chronic lung inflammatory diseases [Reviewed in (24; 25; 29)]. Reactive oxygen species (ROS) are highly unstable molecules produced by the pulmonary epithelial and endothelial cells involved in many forms of tissue damage including the damage caused to cellular components such as lipids, proteins, and DNA [Reviewed in (1; 10)]. We have previously shown that RSV infection of airway epithelial cells induces ROS production, which is involved in transcription factor activation and chemokine gene expression (6; 23). We have also shown that rapid generation of ROS are associated with oxidative stress and lung damage in infected cells in both animals and children (7; 14; 15). Antioxidant treatment significantly ameliorates RSV-induced oxidative stress, clinical disease and pulmonary inflammation in a mouse model of infection, suggesting a causal relationship between increased ROS production and lung disease (7). RSV infection leads to a significant decrease in the expression and activity of antioxidant enzymes (AOEs) in airway epithelial cells (AECs) in lungs of RSV-infected mice, as well as in children with severe RSV-induced lower respiratory tract infection (LRTI) (14; 15), suggesting that oxidative damage associated with RSV infection results from an imbalance between ROS production and antioxidant cellular defenses.

The use of recombinant SOD and SOD mimetics has been explored as therapeutics in a variety of disease models either *in vitro* or *in vivo*. A number of SOD mimetics based around organo-manganese complexes have been developed. They include metalloporphyrin-based compounds, such as AEOL10113 and 10150, cyclic polyamine-based molecules, such as M40403 and 40419, and the salen compounds, such as EUK-8, -134 and -189, the latter ones possessing also significant catalase and peroxidase activity [Reviewed in (3)]. Although EUKs have been used in a variety of disease models, there is no reported literature about their use in models of viral infections. Recently, we have shown that treatment of A549 cells with EUK-134 significantly inhibits RSV-induced IL-8 and RANTES secretion (15). In this study, we found that EUK-8 and EUK-189 treatment of AECs significantly restored intracellular catalase and glutathione peroxidase enzyme activities significantly diminished by RSV infection, leading to reduced viral-induced ROS production and generation of lipid peroxidation markers, such as isoprostane and MDA. In addition, EUK administration significantly reduced secretion of a variety of pro-inflammatory molecules, besides IL-8 and RANTES, in response to RSV infection. At high concentration, both EUK-8 and -189 reduced viral replication, indicating that EUKs could represent a novel therapeutic approach to restore the pro-oxidant/antioxidant balance in favor of the latter, in the context of RSV infection, leading to reduced cellular oxidative stress, pro-inflammatory mediator secretion and reduced viral replication.

MATERIALS AND METHODS

Materials. Eukarion compounds (salen-manganese complexes) EUK-8 and EUK-189 were kindly provided by Susan Doctrow (Boston University, School of Medicine). 2′,7′-

dichlorodihydro-fluorescein diacetate (DCF-DA) was from Invitrogen, Molecular Probes Division, Eugene, Oregon, USA; 3-Amino-9-Ehtyl Carbazole from Sigma, St. Louis, MO.

RSV preparation. The RSV Long strain was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere (34). The virus titer of the purified RSV pools was 8-9 $\log_{10}$ plaque forming units (PFU)/mL using a methylcellulose plaque assay. No contaminating cytokines were found in these sucrose-purified viral preparations (27). LPS, assayed using the limulus hemocyanin agglutination assay, was not detected. Virus pools were aliquoted, quick-frozen on dry ice/alcohol and stored at -70^{o}C until used.

Cell culture and infection of epithelial cells with RSV. A549 cells, a human alveolar type II-like epithelial cell line (American Type Culture Collection, Manassas, VA) and small alveolar epithelial (SAE) cells (Clonetics, San Diego, CA), normal human airway epithelial cells derived from terminal bronchioli, were grown according to the manufacturer's instructions. A549 and SAE were maintained in F12K and small airway epithelial cell (SAEC) growth medium respectively, containing 10% (vol/vol) FBS, 10 mM glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin for F12K medium, and 7.5 mg/mL bovine pituitary extract (BPE), 0.5 mg/mL hydrocortisone, 0.5 µg/mL hEGF, 0.5 mg/mL epinephrine, 10 mg/mL transferrin, 5 mg/mL insulin, 0.1 µg/mL retinoic acid, 0.5 µg/mL triiodothyronine, 50 mg/mL gentamicin and 50 mg/mL bovine serum albumin (BSA) for SAEC medium. When SAE were used for RSV-infection, they were changed to basal medium, not supplemented with growth factors, 6 h prior to and throughout the length of the experiment. At around 80-90 % confluency, cell monolayers were infected with RSV at multiplicity of infection (MOI) of 3 (unless otherwise stated), as

previously described (11). An equivalent amount of a 30% sucrose solution was added to uninfected A549 and SAE cells, as a control.

For the catalytic scavenger experiment, cells were pretreated with EUK-8 or EUK-189 for one hour and then infected in the presence of the compound. Since EUKs were diluted in ethanol, equal amounts of ethanol were added to untreated cells, as control. Total number of cells and cell viability, following antioxidant treatment, were measured by trypan blue exclusion. There was no significant change in cell viability with both compounds at all doses tested.

Antiviral assay in the presence of EUKs. A549 cells were seeded into 48-well plates at $5x10^4$ cells per well. Cells were treated with UEKs in triplicate wells for 1 h and infected with RSV at a MOI of 0.01 PFU/cell. Following adsorption of virus for 1 h at 37°C and 5% CO_2, viral inoculum was aspirated, the cells washed three times with MEM, then MEM with 2% FBS added. Control or EUK-treated and infected cells were then incubated at 37°C and 5% CO_2 for 24 h. RSV titers were determined by using polyclonal antibodies and an HRP staining method, as previously described (20). Briefly, medium was aspirated and the cells fixed for 20 min using methanol and 2% H_2O_2. Cells were then incubated for 30 min with anti-RSV polyclonal antibody (Biogenesis, Kingston, New Hampshire) followed by HRP-conjugated anti-guinea pig secondary antibody (Zymed, San Francisco, CA). Plaques were visualized by the addition of 3-amino-9-ethyl-carbazole (AEC) substrate and enumerated by light microscopy.

Measurement of intracellular reactive oxygen species. A549 cells were grown in 96-well tissue culture plates and infected with RSV. At different times post-infection, cells were washed with Hank's Balanced Salt Solution (HBSS) and loaded with 10 μM 2,7 DCF-DA in HBSS medium

containing 25 mM HEPES, pH 7.4, for 30 minutes at 37°C. The cells were then washed twice and fluorescence intensity was determined at 485 nm excitation and 590 nm emission, using an automated fluorescence reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of lipid peroxidation products. Measurement of F_2 8-isoprostane was performed using a competitive enzyme immunoassay from Cayman Chemical, Ann Arbor, MI, according to manufacturer's instructions. Measurement of lipid peroxidation markers MDA was carried out using a lipid peroxidation kit from Calbiochem/EMD Chemicals, USA.

Western blotting. Nuclear extracts of uninfected and infected cells were prepared using hypotonic/nonionic detergent lysis, according to Schaffner protocol (31). To prevent contamination with cytoplasmic proteins, isolated nuclei were purified by centrifugation through 1.7 M sucrose buffer for 30 minutes, at 12,000 rpm, before nuclear protein extraction, as previously described (5). Total cell lysates were prepared from uninfected and infected A549 cells by adding ice-cold lysis buffer (50 mM Tric-HCl, pH 7.4, 150 mM NaCl, 1mM EGTA, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1% Triton X-100 and 1 μg/ml of aprotinin, leupeptin and pepstatin). After incubation on ice for 10 min, the lysates were collected and detergent insoluble materials were removed by centrifugation at 4^o C at 14,000 g. Proteins (10 to 20 μg per sample) were then boiled in 2X Laemmli buffer and resolved on SDS-PAGE. Proteins were transferred onto Hybond-polyvinylidene difluoride membrane (Amersham, Piscataway, NJ) and nonspecific binding sites were blocked by immersing the membrane in Tris-buffered saline-Tween (TBST) containing 5% skim milk powder or 5% bovine serum albumin for 30 minutes. After a short wash in TBST, membranes were incubated with the primary

antibody for 1 h or overnight at 4^{o}C, depending on the antibody used, followed by HRP-conjugated secondary antibody (Sigma, St. Louis, MO, diluted 1:10,000 in TBST for 30 min at room temperature. After washing, proteins were detected using an enhanced chemiluminescence system (Amersham Life Science, Piscataway, NJ) and visualized through autoradiography. Antibodies used for Western blot assay are, goat anti-RSV, from Ab D SeroTec, rabbit anti-p65, anti-Ser536 p65 and anti-IRF-3, from Cell Signaling Technology, Inc, Danvers, MA.

Biochemical assays. Catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were determined using specific kits (Cayman Chemical; Catalog No. 707002, 703102 and 706002, respectively, for catalase, GPx and SOD), according to the manufacturer's instructions, as previously described (15).

Bio-Plex. Cell-free supernatant from EUK-8 and EUK-189-treated and virus- and mock-infected A549 and SAE cells were collected at 24 h p.i. to measure the production of cytokines and chemokines. Samples were tested for multiple cytokines using the Bio-Plex Cytokine Human Multi-Plex panel (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. IL-8 and RANTES were also quantified by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (DuoSet R&D Systems, Minneapolis, MN).

Statistics. A two tailed student's t-test using a 95% confidence levels were performed on all experiments. Significance is designated by the following: * $p<0.05$, ** $p<0.01$

RESULTS

Antioxidant mimetic, EUK-8 increases total SOD, catalase and GPx enzyme activities in RSV-infected A549 cells. In recent investigations, we have shown that RSV infection of AECs induces a significant decrease in SOD 1, SOD 3, catalase and GST expression with a concomitant increase of SOD 2. Total SOD activity was increased but catalase, glutathione peroxidase (GPx) and GST activities, needed to detoxify H_2O_2 produced by SOD, were decreased following RSV infection (15). In this study we tested whether treatment with the antioxidant mimetics EUK-8 and -189, which possess significant catalase and peroxidase activity, in addition to SOD, could restore antioxidant enzyme capacity in RSV-infected airway epithelial cells and thereby exert a protective effect against RSV-induced oxidative stress.

A549 cells were treated with increasing concentration of EUK and infected with RSV. Cells were harvested at different time p.i. to measure AOE activity in the presence or absence of EUK treatment. RSV infection induced a progressive increase in SOD activity with a concomitant decrease in catalase and peroxidase activity (Fig.1). EUK-8 treatment further increased SOD activity but, more importantly, reversed the loss of catalase and peroxidase activity observed in response to RSV infection, with the highest dose of EUK-8 increasing the latter two AOE activities above values of uninfected cells (Fig.1). Similar results were obtained in cells treated with EUK-189 (data not shown).

Effects of antioxidant mimetics on RSV-induced ROS formation and cellular oxidative stress. To determine whether EUK treatment could reduce RSV-induced ROS production and cellular oxidative stress, A549 cells were treated with different concentrations of the two antioxidant mimetics, infected with RSV and harvested to measure ROS generation and concentration of the

lipid peroxidation markers MDA and F_2-8 isoprostane. As previously reported (6), RSV infection of airway epithelial cells induced a time-dependent increase in ROS generation, starting between 1 and 3 h p.i. (Fig. 2A), which was significantly reduced by treatment with both EUK-8 and EUK-189, in a dose-dependent manner (Fig. 2B). In agreement with the observed reduction in ROS production, EUK treatment of airway epithelial cells significantly reduced the elevated cellular levels of the lipid peroxidation markers MDA and 8-isoprostane generated in response to RSV infection. This effect was observed even at the lowest dose of EUK used (Fig. 3), indicating that antioxidant mimetic treatment can effectively counteract viral-induced cellular oxidative stress.

Effects of antioxidant mimetics on RSV-induced cellular signaling. As ROS generation plays a key role in RSV-induced cellular signaling, leading to transcription factor activation and expression of pro-inflammatory mediators (6; 18; 23), we investigated the effect of EUK treatment on viral-induced cytokine and chemokine secretion. A549 cells were treated with increasing concentrations of EUK-8 and -189 and infected with RSV. Cells supernatants were collected to measure levels of various cytokine and chemokines by Bio-Plex assay. As shown in Fig. 4A, EUK administration caused a dose-dependent decrease in several cytokines, such as IL-6 and G-CSF, and chemokines, such as IL-8, RANTES, MIP-1β and IP-10. Similar results were obtained in SAE cells, normal human airway epithelial cells derived from cadaveric donor, which we have previously shown to behave very similarly to A549 cells in terms of chemokine/cytokine gene expression, transcription factor and signaling pathway activation, after RSV infection (2; 6; 11; 14; 26; 28; 35)(Fig. 4B).

Cytokine and chemokine gene expression in AECs infected by RSV is orchestrated by activation of two key transcription factors, NF-κB and IRF-3. A number of RSV-inducible inflammatory and immunoregulatory genes require NF-κB for their transcription and/or are dependent on an intact NF-κB signaling pathway (4; 33), and IRF-3 is necessary for viral induction of RANTES transcription and gene expression (22). We have recently shown that treatment of airway epithelial cells with the antioxidant butylated hydroxyanisole (BHA) blocks RSV-induced IRF-3 nuclear translocation and DNA binding to the RANTES ISRE (6), an event required for RSV-induced RANTES gene transcription. We have also shown that the antioxidants N-acetyl cysteine or dimethyl sulfoxide significantly reduce RSV-dependent serine phosphorylation of the NF-κB subunit p65, resulting in the inhibition of RSV-induced expression of several NF-κB-dependent genes, without affecting nuclear translocation (18). To determine whether EUK treatment was able to modulate viral-induced NF-κB and IRF-3 activation, A549 cells were treated with 100 μM EUK-8, infected with RSV and harvested at 6 and 15 h p.i. to prepare either total cell lysates or nuclear extracts. IRF-3 nuclear levels or cellular levels of p65 serine phosphorylation were assessed by Western blot. As shown in Fig. 5, EUK-8 treatment significantly reduced activation of both transcription factors, in particular at the 15 h time point of infection. Taken together, these results indicate that antioxidant mimetic treatment can effectively modulate the strong pro-inflammatory cellular response induced by this viral infection.

Effects of antioxidant mimetics on viral replication. To determine whether antioxidant mimetic treatment of A549 cells affected viral replication, we used two different approaches, the first employing a direct cell-based plaque immunostaining assay (20), while the second used viral

antigen detection by Western blot. As shown in Fig. 6A, there was no significant difference in the number of plaques between untreated and EUK-treated cells at the two lower concentrations (10 and 100 μM), while the highest one, both in EUK-8 and -189-treated cells, led to a significant reduction in the number of plaques detected by immunostaining of infected cells.

To further characterize the antiviral activity of EUKs, we assessed the expression of RSV proteins by Western blot. A549 cells were treated with different concentrations of EUKs and infected with RSV. At 24 h post-infection, cells extracts were prepared and RSV proteins were detected by Western blot using a polyclonal antibody, as described (20). In RSV-infected cells, viral proteins, including G, N, P, and M, were expressed at comparable levels in untreated and EUK-treated cells using concentrations of 10 and 100 μM, whereas significant lower (EUK-189) or almost no expression (EUK-8) of RSV proteins was detected in infected cells treated with 500 μM of both compounds (Fig. 6B), confirming the data obtained by direct plaque assay staining.

DISCUSSION

Free radicals and reactive oxygen species have been shown to function as cellular signaling molecules influencing a variety of molecular and biochemical processes, including expression of pro-inflammatory mediators, such as cytokines and chemokines [Reviewed in (1)]. However, excessive ROS formation can lead to a condition of oxidative stress, which has been implicated in the pathogenesis of several acute and chronic airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [Reviewed in (8; 9)]. Inducible ROS generation has been shown following stimulation with a variety of molecules and infection with certain viruses like HIV, Hepatitis B, influenza and rhinovirus [Reviewed in (32)]. In the past few years we have shown that RSV infection of AECs induces ROS production, in part through an NAD(P)H

oxidase-dependent mechanism, inducing oxidative stress *in vitro* (15) and *in vivo* (7), which correlates with severity of disease (14), and that antioxidant treatment blocks transcription factor activation and chemokine gene expression *in vitro* (6; 16; 23) and ameliorates RSV-induced clinical illness *in vivo* (7), indicating a central role of ROS in RSV-induced cellular signaling and lung disease. RSV infection leads to a significant decrease in the expression and activity of AOEs in AECs, lungs of RSV-infected mice, as well as in children with severe RSV-induced lower respiratory tract infection (LRTI)(14; 15), likely due to decreased activation of NF-E2-related factor 2 (Nrf2)(14; 15) , which regulates basal and inducible expression of AOE genes (17), suggesting that oxidative damage associated with RSV infection results from an imbalance between ROS production and antioxidant cellular defenses.

Based on this strong supportive evidence that RSV-induced intracellular ROS formation regulates the expression of proinflammatory mediators and that oxidative stress likely represents an important pathogenetic mechanism of RSV-induced lung disease, antioxidant intervention would represent a rational approach for treatment of RSV lower respiratory tract infections. Two complementary approaches could be used to affect the outcome of RSV-associated lower respiratory tract infections. The first would be to increase airway antioxidant defenses by modulation of AOE expression/activity, and the second would be by enhancing non-enzymatic defenses through pharmacological intervention with molecules able to scavenge/detoxify ROS. Approaches that combine scavenging ROS by administration of antioxidant compounds or compounds able to increase lung antioxidant defenses, such as AOE mimetics or Nrf2 inducers, together with inhibitors of viral replication would likely be the most effective in modulating severe lung disease associated with RSV infection.

In the past few years several classes of synthetic antioxidant mimetics have been generated and tested as a potential therapeutic approach to oxidant-related lung damage. The salen class of AOE mimetics includes compounds that have mainly SOD activity as well as compounds that, in addition, exhibit catalase and peroxidase activity. These molecules have been shown to be effective in preventing lung injury in animal models of oxidative stress, as well as to protect against damage of other organs, such as heart, kidney and liver [Reviewed in (19)]. EUK-8 administration has been shown to ameliorate LPS-induced lung injury in a porcine model of endotoxemia (12; 13) and to mitigate lung radiation injury (30). In this study, we found that treatment of AECs with EUK-8 and -189 effectively restored catalase and glutathione peroxidase enzyme activities, which were significantly decreased in response to RSV infection, leading to reduced viral-induced ROS production and generation of the lipid peroxidation markers isoprostanes and MDA, as well as reduced activation of the ROS-dependent signaling pathway involved in NF-κB and IRF-3 activation and pro-inflammatory gene expression. This is the first report of the effect of EUKs on oxidative stress in a model of viral infection. We had previously reported that treatment of epithelial cells with EUK-134, which is similar to EUK-8 and -189, but not EUK-163, which lacks catalase or peroxidase activity, significantly inhibited RSV-induced IL-8 and RANTES secretion (15). This suggests that enhancement of cellular SOD activity alone in response to RSV infection cannot modulate ROS-mediated signaling and subsequent viral-induced gene expression, while increasing the levels of catalase and/or peroxidase activity is beneficial in reducing pro-inflammatory gene expression.

In addition, both EUKs were able to significantly reduce viral replication, which also represents a novel finding for this type of antioxidant compounds. We did not observe a similar effect when AECs were treated with other classes of antioxidants, including BHA (6), N-acetyl

cysteine or dimethyl sulfoxide (18). Future studies will elucidate the mechanism(s) of EUK antiviral activity. These results, together with our previous finding that antioxidant treatment attenuates symptoms and pathology in RSV infection (7), warrant further investigation of AOE mimetics as a novel therapeutic approach to modulate viral-induced pulmonary disease. Antioxidant supplementation would be successful only if available at the site of infection/inflammation, therefore route of administration, bioavailability, tissue distribution are all important parameters that will need to be taken into consideration when planning future therapeutic intervention.

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AUTHOR CONTRIBUTION

Conceived and designed the experiments: AC, RPG. Performed the experiments: YMH, NM, TL. Analyzed the data: YMH, AC, RPG. Wrote the paper: AC, RPG.

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FIGURE LEGENDS

Fig. 1. Effect of EUK treatment on total SOD, catalase and GPx activity in RSV-infected cells. Total lysates were prepared from uninfected and RSV-infected treated or untreated with different doses of EUK-8 treated at 6, 15 and 24 h p.i. to measure total SOD, catalase and GPx enzyme activities. Data are expressed as percent change over control. Results are representative of two independent experiments.

Fig. 2. Effect of EUK treatment on RSV-induced ROS formation and cellular oxidative stress. (**A**) A549 cells were infected with RSV and, at various time points after infection, cells were loaded with DCF-DA and fluorescence was measured in control and infected cells, * $p<0.05$, ** $p<0.01$ compared to control cells. (**B**) A549 cells were treated with different micromolar concentrations of EUK-8 and EUK-189, infected with RSV for 24 h, and harvested to measure DCF-DA fluorescence. Ctrl indicates control, uninfected cells. Mean Fluorescence Intensity is reported as percent increase over control. Results are representative of two independent experiments. * $p<0.05$, ** $p<0.01$ compared to untreated RSV-infected cells.

Fig. 3. Effect of EUK treatment on RSV-induced lipid peroxidation. A549 cells were treated with different micromolar concentrations of EUK-8 and EUK-189 and infected with RSV. Cell supernatants were harvested at 24 h p.i. to measure F2-isoprostanes and MDA. Ctrl indicates control, uninfected cells. Results are expressed as mean ± standard error. Results are representative of two independent experiments run in triplicate. * $p<0.05$, ** $p<0.01$ compared to untreated RSV-infected cells.

Fig. 4. Effect of EUK treatment on RSV-induced cytokines and chemokines production. A549 cells (**A**) or SAE cells (**B**) were infected with RSV in the absence or presence of different micromolar concentrations of EUK-8 and EUK-189. Cell supernatants from uninfected and RSV-infected, treated or untreated, were assayed at 24 h p.i. for cytokines and chemokines secretion by Bio-Plex. Ctrl indicates control, uninfected cells. Results are expressed as mean ± standard error. Results are representative of two independent experiments run in triplicate. * $p<0.05$, ** $p<0.01$ compared to untreated RSV-infected cells.

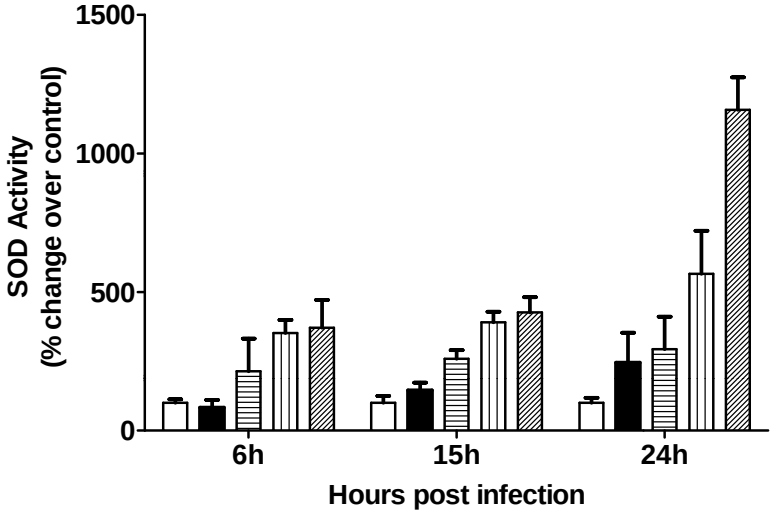
Fig.5. Effect of EUK treatment on RSV-induced IRF-3 and p65 activation. Total cell lysates (**A**) or nuclear extracts (**B**) were prepared from A549 cells control and infected with RSV for 6 and 15 h, in the absence or presence of 100 μM EUK-8, and assayed for p65 phosphorylation and IRF-3 nuclear levels, respectively, by Western blot. Membranes were stripped and reprobed for either β-actin or lamin b as a control for equal loading of the samples. Blot is representative of two independent experiments with similar results.

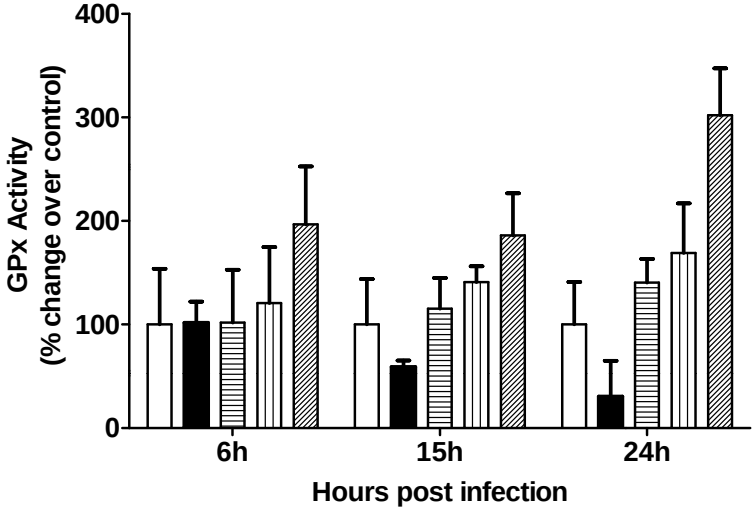
Fig. 6. Effect of EUK treatment on viral replication. (**A**) A549 cells were treated with different micromolar concentrations of EUK-8 and EUK-189 followed by infection with RSV at an MOI of 0.01. Viral replication was determined 24 h p.i. by direct HRP-staining plaque assay (see Methods). Ctrl indicates uninfected cells. Data is representative of two independent experiments with similar results. ** $p<0.01$ compared to untreated RSV-infected cells. (**B**) A549 cells were treated with different micromolar concentrations of EUK-8 and EUK-189, followed by infection with RSV at an MOI of 3. Cells were harvested at 24 h p.i. to prepare total cell lysates and RSV proteins were detected by western blot using an anti-RSV polyclonal antibody.

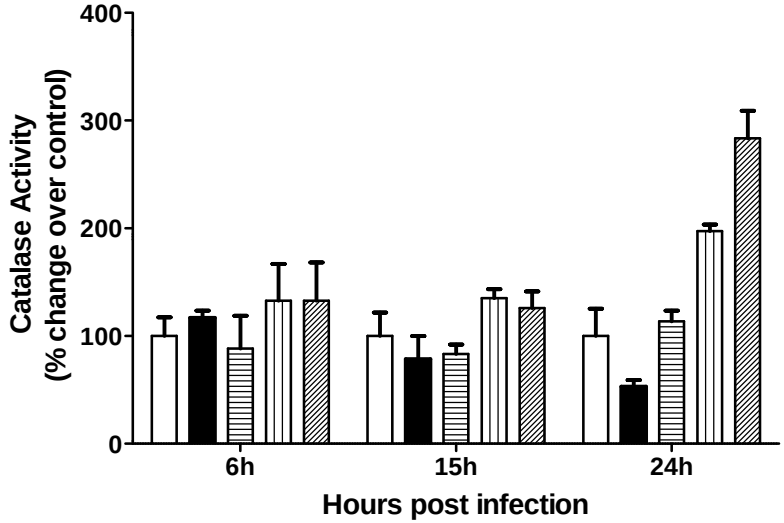
Membrane was stripped and reprobed with β-actin as a control for equal loading of the samples.

Blot is representative of two independent experiments with similar results.

Fig.1


SOD Activity
(% change over control)
1500
1000
500
0
6h
15h
24h
Hours post infection


GPx Activity
(% change over control)
400
300
200
100
0
6h
15h
24h
Hours post infection


Catalase Activity
(% change over control)
400
300
200
100
0
6h
15h
24h
Hours post infection

Control
RSV
EUK-8 10uM
EUK-8 100uM
EUK-8 500uM

Fig.2

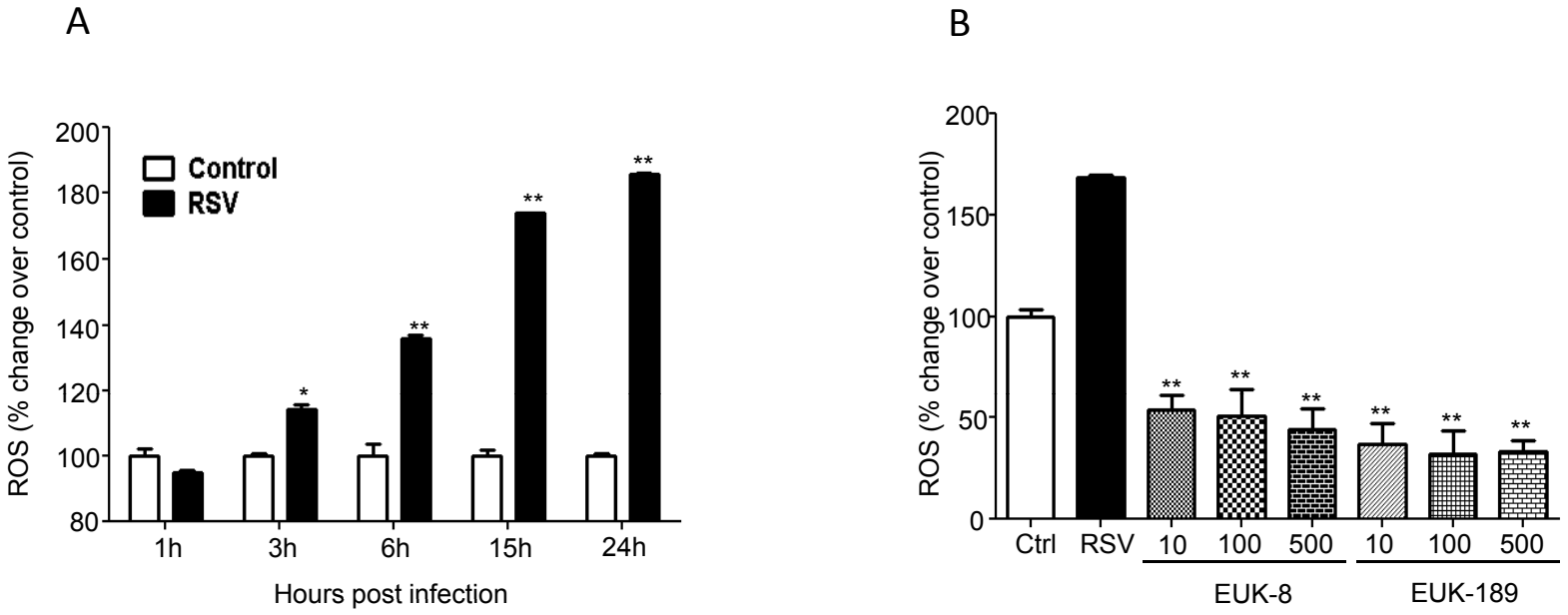

A
ROS (% change over control)
200
180
160
140
120
100
80
Control
RSV
*
**
**
**
1h
3h
6h
15h
24h
Hours post infection
B
ROS (% change over control)
200
150
100
50
0
**
**
**
**
**
**
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189

Fig.3

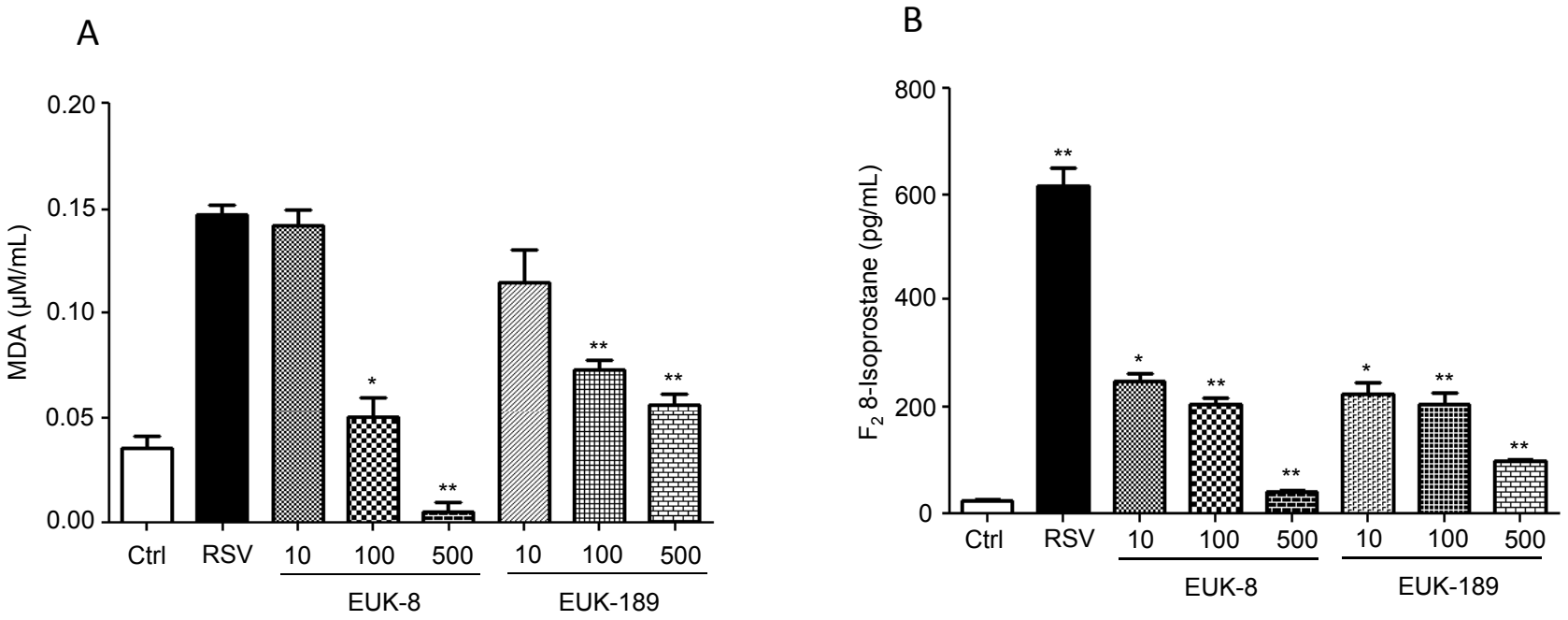

A
MDA (μM/mL)
0.20
0.15
0.10
0.05
0.00
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189
B
F_2 8-Isoprostane (pg/mL)
800
600
400
200
0
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189

Fig.4A

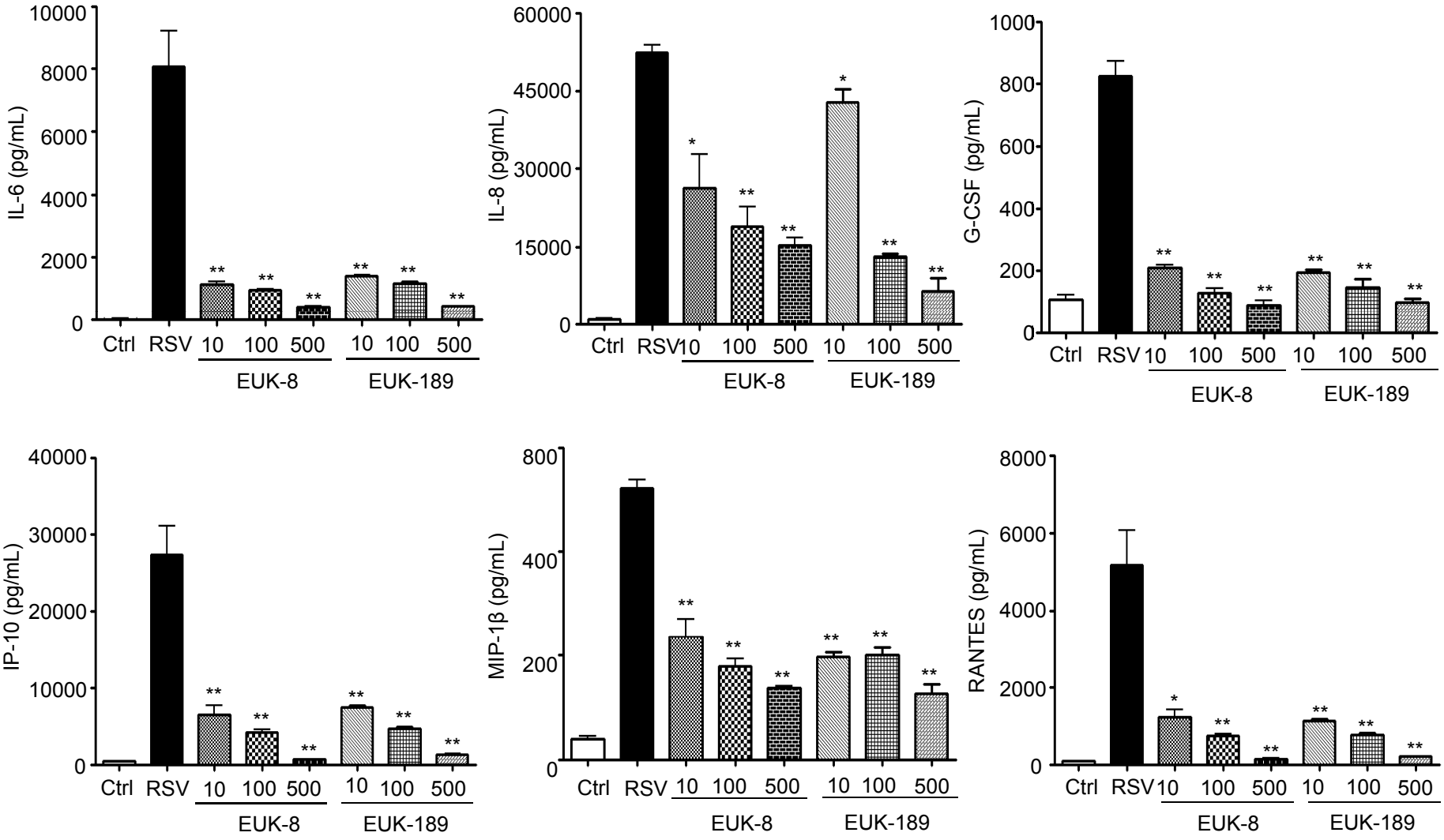

IL-6 (pg/mL)
10000
8000
6000
4000
2000
0
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189
IL-8 (pg/mL)
60000
45000
30000
15000
0
G-CSF (pg/mL)
1000
800
600
400
200
0
IP-10 (pg/mL)
40000
30000
20000
10000
0
MIP-1β (pg/mL)
800
400
200
0
RANTES (pg/mL)
8000
6000
4000
2000
0

Fig.4B

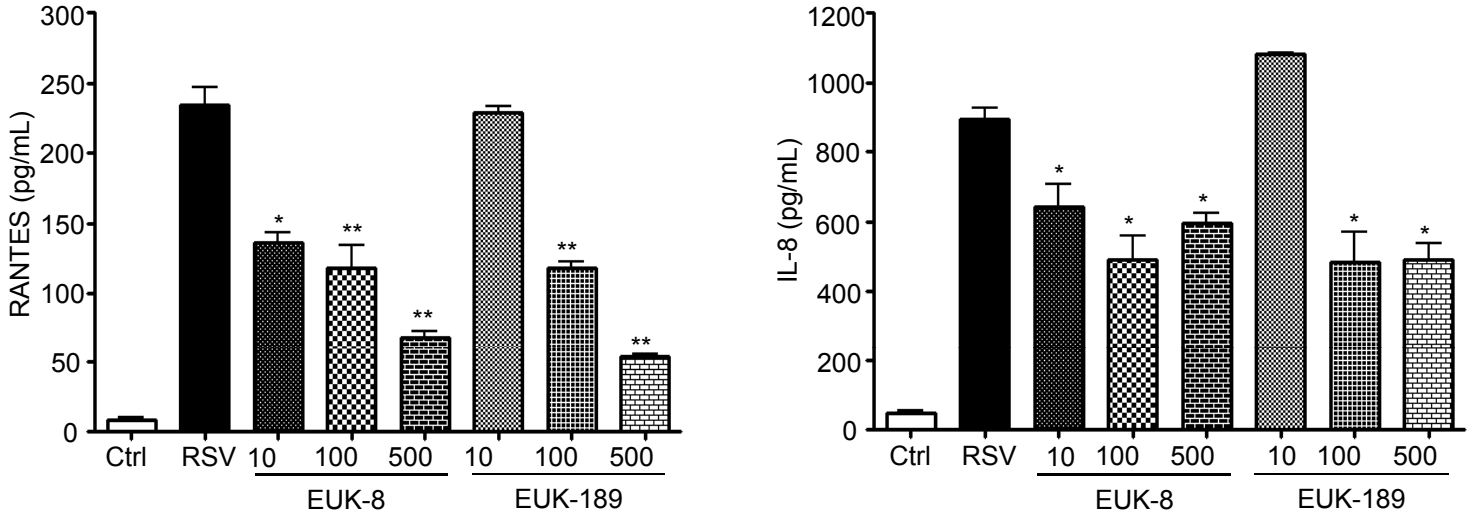
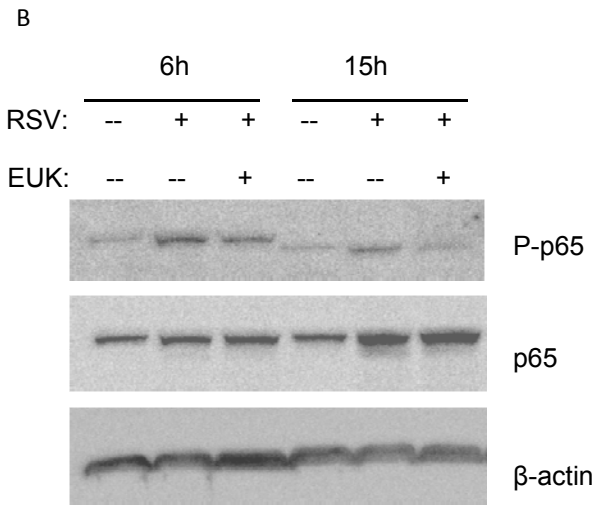

RANTES (pg/mL)
300
250
200
150
100
50
0
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189
*
**
**
**
**
IL-8 (pg/mL)
1200
1000
800
600
400
200
0
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189
*
*
*
*
*

Fig.5



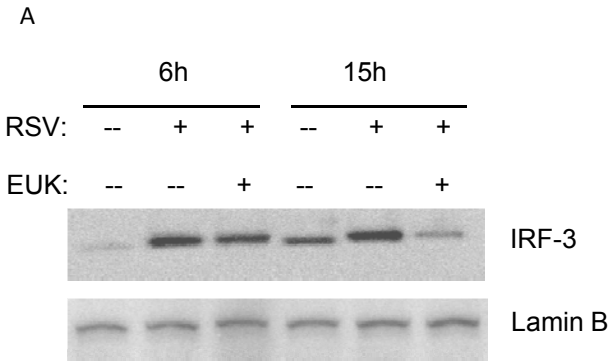
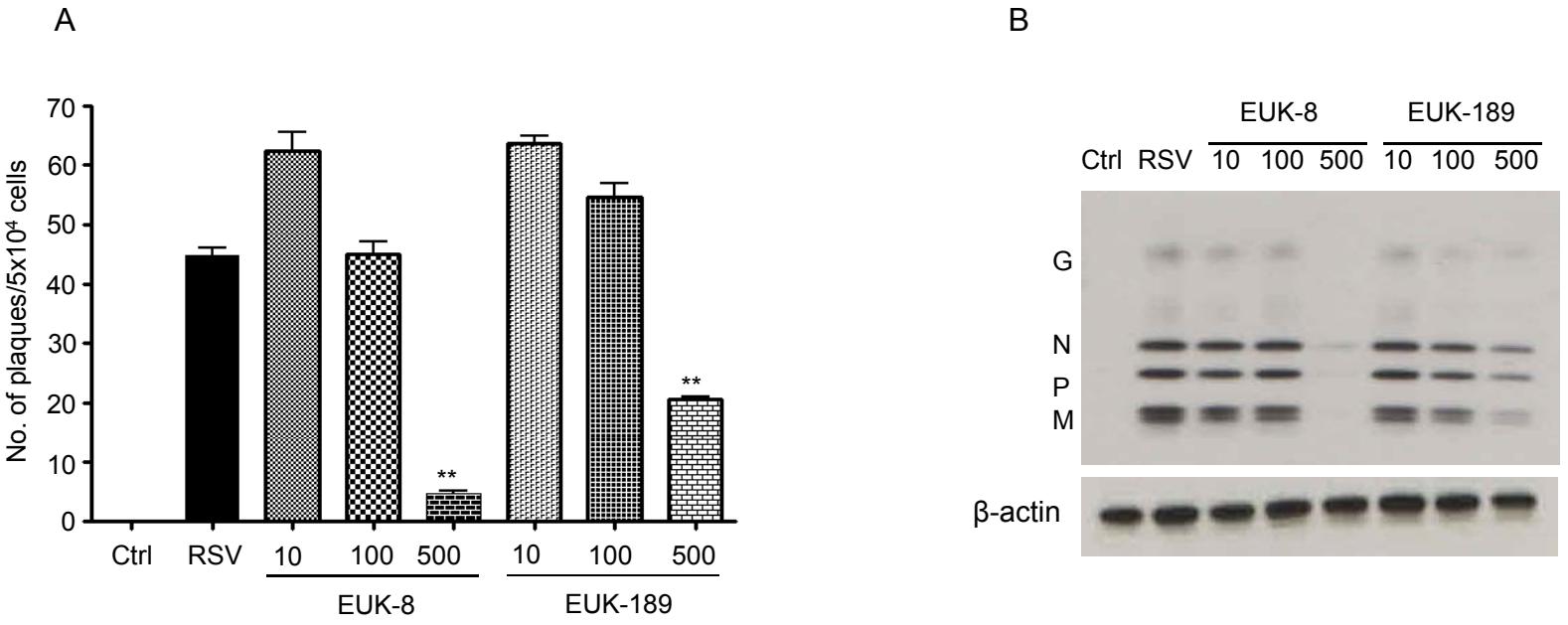


Fig.6


A
No. of plaques/5x10⁴ cells
70
60
50
40
30
20
10
0
**
**
Ctrl
RSV
10
100
500
10
100
500
EUK-8
EUK-189
B
EUK-8
EUK-189
Ctrl
RSV
10
100
500
10
100
500
G
N
P
M
β-actin